EXHIBIT H

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A Sensitive and Quantitative Element-Tagged Immunoassay with ICPMS Detection

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We report a set of novel immunoassays in which proteins of interest can be detected using specific element-tagged antibodies. These immunoassays are directly coupled with an inductively coupled plasma mass spectrometer (ICPMS) to quantify the elemental (in this work, metal) component of the reacted tagged antibodies. It is demonstrated that these methods can detect levels of target proteins as low as 0.1–0.5 ng/mL and yield a linear response to protein concentration over 3 orders of magnitude.

We report an attempt to measure directly the atomic composition of a tag conjugated to a biologically active material. There are many methods of tagging biologically active materials that can provide analysis with high signal-to-noise ratio using different physical methods of detection. Because tags specially designed for elemental analysis are not yet developed, we have used a variety of immunoreagents that already include metals for different purposes. In the following few paragraphs, we discuss several such methods and reagents that may be of particular significance.

Colloidal gold or extremely small gold clusters (<2 nm in diameter) are extensively used to visualize protein structure in the cell and to detect receptor–ligand binding by electron microscopy.[1-5] To the best of our knowledge, elemental analysis (employing electrothermal atomic absorption spectrometry, ICPMS) of conjugated gold clusters was first reported for the determination and quantitation of low-density lipoprotein[6-8] and in the context of an immunoassay with ICPMS detection in ref 9. In this paper, we will demonstrate that gold-cluster antibody conjugates can also be successfully used in immunoassays with determination of the elemental tag using an ICPMS. Elemental nanoparticles are especially attractive for quantitation because of their uniform size and significant number of atoms per conjugate. There is also the possibility of increasing the signal response yet further by using silver enhancement.[5] Tungsten, silver, and platinum[10-12] might also be considered as elemental tags but are not yet commercially available.

Releasable fluorescent probes[13,14] have been discussed extensively and have been marketed for use in direct and competitive immunoassays (Wallac AutoDELFIA[15]). Lanthanide ions have been complexed to a wide spectrum of molecules, and the fluorescence of their chelates provides a sensitive means of detection. The Wallac AutoDELFIA system utilizes the fluorescent properties of four lanthanides chelates (Eu, Tb, Dy, and Sm) to measure the concentrations of various antigens in an automated immunoassay system. These are a set of fluoroimmunometric assays based on direct, competitive, and sandwich enzyme linked immunosorbent assay (ELISA) methods. In each assay, lanthanide-labeled antibodies (or competing peptides) are reacted with the antigen of interest and then quantified by the addition of chelators, which extract the lanthanide ions from the antibodies (or peptides) to form fluorescent chelates. The Wallac AutoDELFIA antibodies are labeled with six to eight atoms of lanthanide.

This approach allows limited (typically, up to four) multianalyte detection, provided that the fluorescence responses can be spectroscopically resolved. In addition, the use of different nanosphere conjugates[14] could enhance the fluorometric signal by providing ~1000 extractable ions per antibody. This feature is also advantageous for mass spectrometric determination of the element of the tag. It should be straightforward to quantify the lanthanide ions directly employing a means of elemental analysis, such as ICPMS. In the case of mass spectrometry, the mass-to-charge ratio of an element contained in a tag provides the potential for multianalyte detection using different isotopes and simplifies the assay by eliminating the chelator.

The quantitation and detection of specific proteins (antigens) in biological samples have traditionally been achieved using Western blotting combined with electrochemiluminescence (ECL). It is a sensitive method and is widely available. Western blotting, however, has several drawbacks and limitations, namely, (i) low amounts of protein may not be detected (<1–2 ng), (ii) the amount of protein is difficult to quantify, and (iii) nonspecific

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Table 1. ICP Operating Conditions

sample introduction	peristaltic pump (from 20 μL to 0.5 mL min^{-1} uptake) MicroFlow PFA-ST concentric nebulizer (ESI) quartz cyclonic spray chamber
RF plasma source	free-running (nominal 40 MHz) ICP 1400 W(typical)
plasma gas	15 L min^{-1} Ar
auxiliary gas	1.2 L min^{-1} Ar
nebulizer gas	1.02 L min^{-1} Ar (optimized for Ir)

binding of both antibodies may result in high background or the appearance of several unexpected bands. Densitometers are commonly used to compare the density of bands to known standards to estimate the concentration of protein present. This method is inaccurate as a result of the loss of some proteins during transfer blotting as well as the nonlinear relationship between ECL and protein concentration. Although this method does not provide tags that are suitable for elemental analysis, it was used for evaluation of antibodies and comparison of detection capabilities.

We will present data showing that four different immunoassays (using centrifugal filtration, protein A affinity, size exclusion gel filtration, or ELISA) can be coupled to the ICPMS to produce a fast and efficient means of detecting proteins of interest with exceptional sensitivity and precision in mixed biological samples.

EXPERIMENTAL SECTION

Apparatus and Materials. Experimental measurements were made on a commercial ELAN 6100 ICPMS (Perkin-Elmer SCIEX), a quadrupole-based mass spectrometer that has been described previously.[16] The inductively coupled plasma (ICP)-generated ions are sampled through a multistage differentially pumped interface, which includes an actively cooled sampler−skimmer stage, an ion optics stage, and a high-vacuum quadrupole mass filter chamber. The ICPMS allows elemental measurements in the mass range 5−250 amu. The operating conditions are summarized in Table 1. The sample uptake rate was adjusted depending on the particular experiment and sample size. A MicroFlow PFA-ST concentric nebulizer (Elemental Scientific, Inc) was used in all instances. Experiments were performed using an autosampler (Perkin-Elmer AS 91) modified for operation with 96 deep well microtiter plates.

Reagents included 1× phosphate buffered saline (PBS; 150 mM NaCl, 20 mM sodium phosphate, pH 7.4), 1% bovine serum albumin (BSA), 10% (volume) HCl (Seastar Chemicals Inc.), buffer A (20 mM Tris, 90 mM NaCl, 2 mM $CaCl_2$, pH 7.5), protein sepharose A (CL-4B, Amersham Pharmacia), human IgG (AG722 Chemicon Lot 20031151), NANOGOLD goat anti-human Fab' conjugate (Fab'−nanoAu; no. 2053, Nanoprobes), Ir and Tl diluted from stock 1000 ppm solutions (SPEX), sephacryl S200 matrix (Amersham Pharmacia), 96-well microtiter plates. Immunology Level 1 Control (no. 07-66230, lot 37459810, ICN) and Immunology Level 2 Control (no. 07-166230, lot 37459811, ICN) were prepared in triplicate in 0.25 mL 1% BSA/PBS. The goat anti-human Fab'−nanoAu reagent consists of affinity-purified Fab' fragments covalently bound to a 1.4-nm nanogold cluster. All solutions were prepared using deionized (Elix/Gradient water purification system, Millipore) distilled water. In the ICPMS-linked filter immunoassay, reaction products were filtered using 300 kDa Centricon (PB-300, Amicon) centrifugal filters (maximum volume, 500 μL and 2 mL). An Eppendorf benchtop centrifuge was used to spin down protein sepharose A reaction products for 10 min at 14 K rpm. An Allegra 21R centrifuge (Beckman Coulter) was used to spin Centricon centrifugal filter devices. A high prep sephacryl column (XK 16, Amersham Pharmacia) was used for ICPMS-linked gel filtration immunoassays.

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RESULTS AND DISCUSSION

Characterization of the Elemental Tag. The gold nanoclusters are regular in size, and if a single cluster is attached to a molecule, one can calculate with high precision the molar concentrations of labeled molecules. Unfortunately, the concentration of commercially available Fab'−nanoAu is not specified exactly by the manufacturer. Specific dilutions are suggested for conventional applications, which do not require absolute quantitation. Thus, iridium is used in this work as an internal standard for quantifying gold concentration. Gold and iridium have close atomic mass numbers and their ionization potentials are similar (9.225 and 9.1 eV, respectively). Thus, the instrumental response is expected to be comparable for both analytes using the ICP ion source. In this work, the ratio of the $^{197}Au/(^{191}Ir + ^{193}Ir)$ ion signals was used to quantify the gold content in samples using standard aqueous solutions containing buffer A acidified (1:1) with 10% HCl and 0.1% HF and spiked with 1 ppb Au and Ir. Both analytes are stable in HCl, and we analyzed only acidified solutions. Gold is known to have a high affinity for surfaces of typical sample introduction systems.[17] However, it was observed[18] that this effect is significantly reduced in the presence of proteins in a sample, probably due to complexation or passivation of surfaces.

The detection limit (DL) was determined as three standard deviations (SD) of the gold signal in the blank solutions (3σ) divided by the net signal in the sample solutions. The DL determined in this manner was 0.1 fmol/mL of Fab'−nanoAu.

Immunoassay, Purification, and Determination of the Dissociation Constant for Antibody−Antigen Complex. A 2-mL portion of 1:500 diluted anti-human Fab'−nanoAu (326 fmol total according to our calibration curve; see Figure 1) was obtained by dilution of stock solution of Fab'−nanoAu in buffer A plus 1 ppb of Ir. The same buffer was later used as a running buffer. The Fab'−nanoAu was filtered using 1%BSA-blocked 300 kDa Centricon 2-mL filters. The filtered Fab'−nanoAu was run through a sephacryl S200 column using the iridium-spiked running buffer. The column was chilled to maintain a temperature of ~8 °C in its cooling jacket. Immediately after exit from the column, the eluate was mixed in the ratio 1:1 with 10% HCl and 0.1% HF plus 1 ppb Tl and run directly into the ICPMS sample introduction system. The analytical method included measurement of ions corresponding to the isotopes ^{197}Au, ^{191}Ir, ^{193}Ir, and ^{205}Tl. The thallium-spiked acidic solution was used in order to keep the sample introduction system (nebulizer and injector) clean and stable. In addition, the

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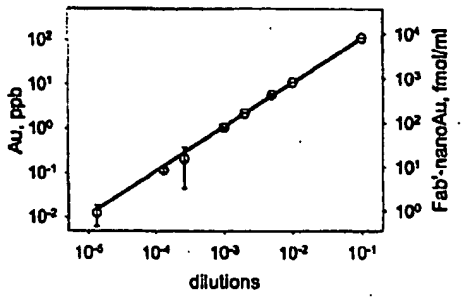

Figure 1. Molar concentrations of serial dilutions of the stock solution of Fab'–nanoAu after filtration through a 300 kDa centrifugal filter. The dilutions were prepared in 1% BSA/PBS and diluted in individual wells of a 96-well microtiter plate with the acidic solution (10% HCl, 0.1% HF spiked with 1 ppb Ir). An estimation of the molar concentrations was made on the assumption that there are 70 atoms of gold in the nanogold cluster. An internal standard of 1 ppb Ir in the acidifying solution was used.

Ir/Tl ratio indicates the proper mixing proportion and overall signal stability. For the period of a typical run, which takes from 2 to 4 h, the instrument drifted less than 10%, as measured by the signals of the analytes of interest. After two runs, the interface cones (sampler and skimmer) were cleaned to restore the instrument sensitivity. The iridium signal indicates the continuity of flow through the column and was used to quantify the gold signal.

Although chromatographically purified by the manufacturer, the Fab'–nanoAu gel filtration results (presented in Figure 2A) revealed the residual presence of unbound nanoclusters and heavier conjugates. For higher purity, a similar run was repeated with a new sample of 2 mL of 1:250 Fab'–nanoAu dilution (652 fmol total) in buffer A plus 1 ppb Ir. An 8-mL portion of the peak fraction of Fab'–nanoAu was collected and not allowed to enter the ICPMS sample introduction system. The concentration of Fab'–nanoAu in this fraction was measured to be 51.3 fmol/mL, or 410 fmol total. One-half of the obtained fraction was run again, and the result is presented in Figure 2B. The second half (4 mL) of the fraction was mixed with 4 μg of human IgG (290 pmol) in buffer A plus 1 ppb Ir, and after a 1-hour incubation at room temperature, it was run through the sephacryl column (see Figure 3).

In this work, two standard errors is used as an estimation of the uncertainties in the estimates of the regression coefficients. For example, fitting of data in Figure 2B leads to an area of 2.63 ± 0.04 ppb*mL which corresponds to ~2.6 ng of gold in the sample. Assuming that there are 70 atoms in the Nanogold cluster, this amount corresponds to ~190 fmol of Fab'–nanoAu in the sample. According to our measurement of concentration in the original solution, the total amount of Fab'–nanoAu is approximately 205 fmol. The weighted fit of Figure 2A data, yields a result of 2.3 ± 0.3 ppb*mL, which corresponds to ≈167 ± 22 fmol of Fab'–nanoAu. The area under the whole peak in Figure 2A is equal to 4.25 ppb*mL (308 fmol). This is somewhat below the expected 326 fmol; however, it is possible to conclude that ~50–60% of the gold signal is not associated with Fab'–nanoAu. Obviously, this conclusion holds only for the stock solution of Fab'–nanoAu used here, and this ratio might be variable. The

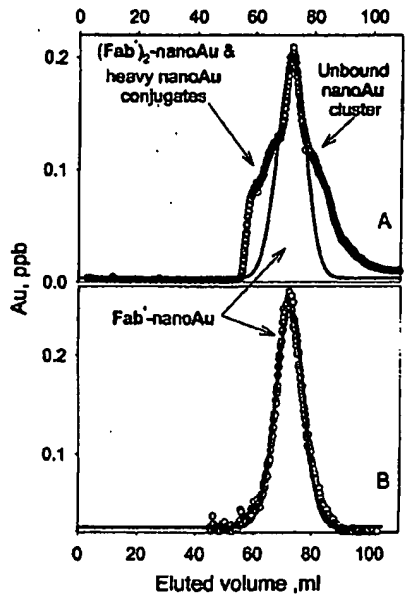

Figure 2. (A) Results of the gel filtration of the Fab'–nanoAu sample. Some contamination with low- and high-mass components is apparent and was attributed to free nanogold and to $(Fab')_2$–nanoAu. The gold signal was detected using ICPMS and quantified by the ratio to the Ir internal standard in running buffer. (B) Gel filtration-purified Fab'–nanoAu. Continuum lines represent the best fit to single Gaussian function. In the case of panel A, the fit was performed with fixed parameters obtained from fitting results on panel B.

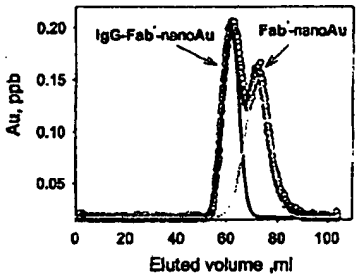

Figure 3. Results of size exclusion filtration of mixture of IgG and Fab'–nanoAu. The quantitation of both the IgG/Fab'–nanoAu complex and free antibody was performed by the best fit of the data to the sum of two Gaussian functions. The continuum lines represent the separate contribution of each analyte (black, IgG-Fab'–nanoAu; gray, Fab'–nanoAu).

manufacturer does not provide sufficient specification for our purposes.

A slightly more complicated (sum of two Gaussians) fitting of the interfered chromatogram in Figure 3 leads to 1.46 ± 0.02 and 1.60 ± 0.03 ppb*mL areas of IgG/Fab'–nanoAu and free Fab'–nanoAu, respectively. The total amount of gold in the sample was 3.06 ± 0.04 ng (222 fmol, as compared with an expected 205 fmol).

Using this technique, one is able to estimate K_d, the dissociation constant (reciprocal of the affinity constant) for the antibody–antigen complex. The binding affinity of the antibody is directly related to the sensitivity (detection limit) of the immunoassay and the linearity of its calibration curves. Two limitations of the

technique include the relatively long period of time it takes for a sample to pass the column and the resolution of the gel filtration. Nevertheless, we used the same column of sephacryl S200 matrix to separate unbound Fab′−nanoAu molecules from IgG/Fab′−nanoAu complexes and to measure the K_d constant. Fab′−nanoAu molecules are ~65 kDa, and IgG/Fab′−nanoAu complexes are ~215 kDa, making separation by gel filtration an attractive alternative to other receptor−ligand binding techniques. The same methods and arrangements for the sample introduction were used. Although we estimate the detection limit for this method to be <10 fmol of Fab′−nanoAu, for this investigation, high concentrations were preferred to increase the signal intensity and improve the accuracy of separation of unbound and bound Fab′−nanoAu.

An 8-mL portion of a 1:20 dilution of the Fab′−nanoAu original solution was run through the column. An ~12 mL portion of the peak fraction of Fab′−nanoAu was collected after gel filtration. Human IgG aliquots of 10 μg each were mixed with 9 serial dilutions of the collected fraction of Fab′−nanoAu, four of them separately and the remaining five in triplicates. The reaction mixtures were gently rocked for 1 h at room temperature, and then the first four samples were run serially through the sephacryl S200 column using the same running buffer, which also included human IgG of the same concentration as that in the samples, allowing the column to be properly equilibrated. Samples were eluted with buffer, on-line mixed with acidifying solution, and analyzed by ICPMS. Results were processed as discussed previously (utilizing the described Gaussian fitting procedure), and the concentrations of IgG/Fab′−nanoAu and Fab′−nanoAu in the effluent were determined. The advantage of this procedure is that only bound IgG can be registered, leaving the unbound portion "invisible" to the detector.

The K_d value calculated via the Scatchard plot for the experimental conditions used was equal to $(2.3 \pm 0.8)\cdot10^{-8}$ M. This value is very important for the characterization of the metal-tagged antibody. The specificity of the final immunoassay is directly related to the affinity of the antibody for the antigen. It is, therefore, paramount to understand that the examples presented here are directly related to the particular antibody used. The ability of the ICPMS technique to detect a metal tag is independent from these specific characteristics.

ICPMS-Linked Filter Immunoassay. The ICPMS-linked filter immunoassay takes advantage of commercially available centrifugal filters to separate bound and unbound antigen−antibody complexes by size. These filters were not originally designed to perform this task. Their ability to discriminate the bound (retained) and unreacted Fab′−nanoAu (filtrate) has not been reported. Potential sources of uncertainty include the difficulty in collecting the retained fraction, unknown nonspecific absorption of filters, distribution of the filter's pore size, and susceptibility (at least, in theory) to blocking by an organic matter of the filter matrix. Further, we do not know how the metal tag interacts with the filter's material and how to extract it from the filter in the most efficient manner. However, it is an attractive method of separation because of its simplicity, speed of analysis, and straightforward automation. The results in Figure 4 depict two calibration curves of human IgG reacted with an excess of prefiltered anti-human Fab′ labeled with Nanogold in 1% BSA/PBS. Anti-human Fab′−nanoAu was prefiltered to remove large

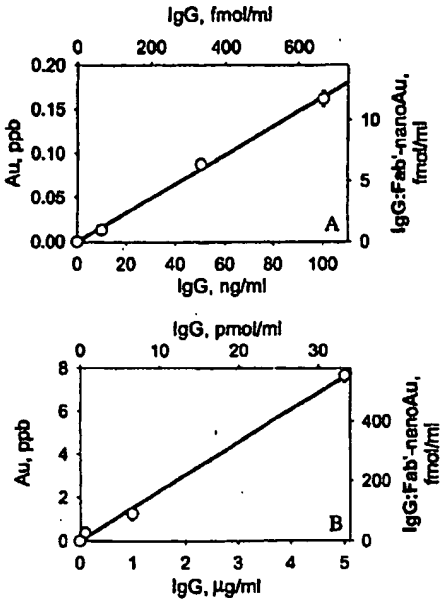

Figure 4. (A,B) Calibration curves of the ICPMS-linked filter immunoassay of human IgG using low (A) and high (B) concentrations of human IgG.

complexes of gold. In panel A, the concentration range of human IgG is 10−100 ng/mL (low range); in panel B, the concentration range of human IgG is 0.1−5 μg/mL (high range). In both cases, an excess of Fab′−nanoAu was provided. We assumed that all IgG/Fab′−nanoAu complexes plus nonspecifically bound Fab′−nanoAu would be retained on the top of the filter and the remainder of unbound Fab′−nanoAu would pass through the filter. Analysis of the blank solution (1% BSA/PBS without IgG, the same content of Fab′−nanoAu) revealed significant retention of the unbound antibody supposedly due to nonspecific absorption on the filter. In both cases, results of analysis of the blank solution were subtracted.

In the presence of an excess of Fab′−nanoAu, one would expect that there is a proportional relationship between the concentration of IgG and the gold detected by the ICPMS. However, as can be seen in Figure 4, the registered molar concentration of the IgG/Fab′−nanoAu complex is significantly lower than the concentration of IgG in the original solution. Such a poor response might indicate that the complex cannot be extracted efficiently from the filter by the usual means. In addition, we have found that the use of a final acid wash was not sufficient to recover gold trapped inside the filter.

In our experiments, the blank solutions (with respect to IgG) contain a significant level of the analyte signal (equivalent to ~7 fmol/mL of the nanoAu clusters), most probably due to nonspecific binding to the filter. As was mentioned before, we used two preventive measures to reduce the nonspecific binding of Fab′−nanoAu to the filter: blocking with 1%BSA/PBS solution and prefiltering of Fab′−nanoAu through the 300 kDa filter (also blocked by the BSA solution). Both steps significantly reduce nonspecific binding. However, it is still a substantial problem for the proposed method. One can conclude that having the gold cluster response (and noise) around 7 fmol/mL in the blank

solution will limit the DL to ≈3 ng/mL (or ≈20 fmol/mL) of IgG; therefore, future viability of this method depends on progress in the development of size-exclusion filters, especially in the 96-well format, specified for 150 kDa cutoff and having very low nonspecific binding toward the tag and tagged materials.

Despite the shortcomings and uncertainties, these results confirm that ICPMS-linked size exclusion filter immunoassays could provide an accurate means of detecting specific antigens over a wide range of concentrations (10−5000 ng/mL); however, high background in the blank solutions might degrade the lower range limit.

ICPMS-Linked Protein Sepharose A Immunoassay. The ICPMS-linked immunoprecipitation assay involves using protein A sepharose (PAS) to immunoprecipitate the target antigen and allow a reaction to occur with anti-human Fab′−nanoAu. All eppendorf tubes, filters, and protein A sepharose beads used were pacified with 1% BSA/PBS. Reaction mixtures were prepared using triplicate serial dilutions of human IgG in the concentration range of 0.1−100 ng/mL (0.667−667 fmol/mL) in 1%BSA/PBS. To detect all IgG present, an excess of both protein A sepharose and anti-human Fab′−nanoAu should be added. The Fab′−nanoAu concentration was selected to be 815 fmol/mL and 0.2% (v/v) protein A sepharose for all IgG dilutions. After incubating aliquots of protein A sepharose with serial dilutions of human IgG for 2 h at room temperature, 815 fmol/ml Fab′−nanoAu was added. The mixtures were incubated for 1 h at room temperature, and then the beads were washed four times with 1% BSA/PBS. The amount of IgG/Fab′−nanoAu complexes bound to the protein A sepharose was measured by digesting the sample in acid and analyzing the amount of gold in the sample using the ICPMS. Results are presented in Figure 5A.

Nonspecific binding limits the dynamic range of this method at low concentration of antigen. According to our previous investigation of K_d for this reaction, we expected that the amount of gold detected would be directly proportional to the amount of PAS/IgG/Fab′−nanoAu complexes formed in the reaction across at least 3 orders of magnitude. However, during this investigation, we found that in order to detect very low amounts of antigen, it is necessary to maintain a low background of gold and a low amount of proteins that form nonspecific binding to Fab′−nanoAu. It is also desirable to prevent binding of Fab−nanoAu to the sides of the eppendorf tubes used for the reaction mixtures. In a separate study, we found that low retention Axygen tubes behaved similar to BSA-pacified eppendorf tubes, which allows us to eliminate one step in the protocol. Still, some deviation from linearity in the low concentration region on the level of 50−10 ppt of gold was observed, and this is largely dependent on the Fab′−nanoAu concentration in the reaction mixture. A low level of contamination with the analyte (a low concentration of antibody in the reaction mixture) should significantly improve the DL at the expense of dynamic range. For this set of experiments (see Figure 5A), the background and sensitivity translate into DL = 0.5 amol/μL for IgG. Therefore, this method should significantly gain from improvements in efficiency of the ICPMS sample introduction system (reduction of sample volume, longer acquisition time) and overall its sensitivity and the suppression of nonspecific binding.

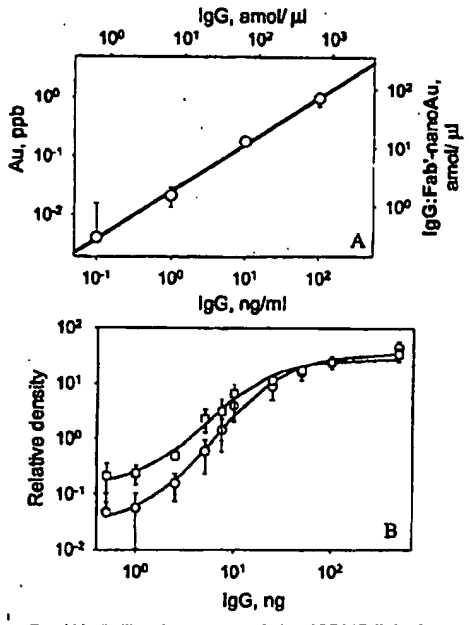

Figure 5. (A) Calibration curve of the ICPMS-linked protein a sepharose immunoassay of human IgG. (B) Results of the quantification of the human IgG using Western blot and densitometry analysis. The results of two different exposures of the same blot are presented. Open circles, 5-s exposure; open squares, 20-s exposure.

It was also evident that linearity failed in the IgG concentration range above 100 ng/mL, which might be expected, because in this range, the molar ratio of antigen and antibody was close to unity, and a shift of the binding equilibrium in the multiple washes in buffer is expected. The obvious solution would be to dilute the antigen or increase the concentration of antibody with resulting degradation in the achievable detection limit.

To further investigate the accuracy and precision of antigen detection through the ICPMS-linked immunoprecipitation assay, we were able to quantify the concentration of a commercially available immunology control material (control serum). In this assay, serial dilutions of human IgG in the concentration range of 0.1−100 ng were prepared in triplicate in 0.25 mL of 1% BSA/PBS. At the same time, four serial dilutions of Immunology Level 1 Control and Level 2 Control were prepared in triplicate in 0.25 mL 1% BSA/PBS. A calibration curve was obtained using dilutions of the human IgG standard. From four serial dilutions of Levels 1 and 2, the amount of IgG present in Level 1 and 2 were determined to be 875 ± 54 and 2303 ± 181 mg/dL, respectively. These determinations rely on the manufacturer's specified concentration of IgG in the standard solution. The uncertainty in this concentration is unknown and was not included in the uncertainties of quantitation of the control serum given above. The manufacturer used four different analyzers to estimate the mean concentration of IgG in Level 1 to be 738 mg/dL (concn range, 590−886 mg/dL) Beckman Array (nephelometric/IFCC RPPHS), 714 mg/dL (concn range, 571−857 mg/dL) BMC Hitachi (IFCC RPPHS), 664 mg/dL (concn range, 531−797 mg/dL) Dade ACA/Star (IFCC RPPHS), and 747 mg/dL (concn range, 598−896 mg/dL) Dade Behring (nephelometric/ IFCC RPPHS). Similarly, the mean concentration of IgG in Level 2 has been estimated to be

2098 mg/dL (concn range, 1678–2518 mg/dL) Beckman Array (nephelometric/IFCC RPPHS), 2109 mg/dL (concn range, 2687–2531 mg/dL) BMC Hitachi (IFCC RPPHS), 1901 mg/dL (conn range, 1521–2281 mg/dL) Dade ACA/Star (IFCC RPPHS), and 2150 mg/dL (concn range, 1720–2580 mg/dL) Dade Behring (nephelometric/IFCC RPPHS).

In a comparison study, we quantified the range of 0.5–500 ng of human IgG using Western blot and densitometry analysis. Western blotting involves using electrophoresis to separate proteins by mass in a polyacrylamide gel. The proteins are then transferred to nitrocellulose using electroblotting. Proteins of interest can be detected in the nitrocellulose blot by probing first with a specific primary antibody raised against the target protein and then using antiprimary, peroxidase-tagged antibodies to detect the protein–antibody complexes. The blot is then exposed to a chemiluminescent substrate and sensitive film paper. Proteins of interest can then be identified by the appearance of bands on the film. Serial dilutions of native human IgG were prepared and loaded into the wells of a 7.5% SDS–polyacrylamide gel, which was run and then transferred to nitrocellulose. The nitrocellulose was blocked and then exposed to anti-human horseradish peroxidase (HRP)-tagged antibody. After washing the blot and soaking it in ECL substrate (Super Signal, Pierce), an imager was used to quantify the relative densities of the IgG bands on the blot. Each blot was exposed for 5- and 20-s intervals. This experiment was performed in triplicate, and a summary of the results is depicted in Figure 5B. It is apparent that 1 ng is the lowest amount of human IgG that could be detected. In both blots, 2.5 ng could be reliably detected at both 5- and 20-s exposures. It is also apparent that the relative densities of the blots do not accurately reflect the concentration of human IgG over the range of concentrations. The expected linear relationship between signal and protein concentration was observed only in the interval 2.5–25 ng of IgG, which might be attributed in part to the limited capability of the imager (256 levels of gray) in data quantification.

It should be noted that the Western blot method has one major advantage over the ICPMS-linked protein A sepharose immunoassay: analyzed quantities of IgG were loaded in a volume of 20 μL. Analyte concentrations were actually from 25 to 2500 ng/mL. In the ICPMS-linked immunoprecipitation assays, we kept the sample volume in the range 0.5–1.5 mL, which was convenient for the standard sample introduction system, with an aspiration rate of approximately 100 μL/min. Miniaturization of the sample introduction system and reduction of the sample uptake rate have the potential to improve the DL of the ICPMS-linked assays.

Atomic Spectroscopy as an Alternative Method of Detection. Nanogold clusters and colloidal gold are not the only metal tags commercially available to date. For example, the AutoDELFIA automatic immunoassay system uses lanthanide-enhanced fluorescent tags. As one step of the validation of the method of determination of metal containing tags using ICPMS, we performed a direct comparison of the ICPMS detection with fluorescent detection using the AutoDELFIA lanthanide-enhanced fluorescent tags. Following immunoprecipitation and separation of the unreacted tagged molecules, ICPMS method depends only on the concentration of the elemental tag. Therefore, the samples may be degraded and stabilized in acid solution for later analysis.

Three AutoDELFIA assays were selected: (1) anti-α-fetoprotein (AFP), (2) anti-β human chorionic gonadotropin (hCG), and (3) anti-estriol. All of the antibodies used for estriol, AFP, and hCG determinations use europium and are thought to contain between 6 and 10 Eu tags/fluorescent tag, and Eu has two isotopes of similar abundance.

The anti-AFP immunoassay is based on the direct sandwich technique, in which the microtiter plate (coated with anti-AFP monoclonal antibodies) is exposed to the sample and europium-labeled anti-AFP antibodies for 1 h. The wells are washed six times, and then a lanthanide chelator (enhancement solution) is added. The anti-hCG immunoassay uses two monoclonal antibodies directed against the two different subunits of hCG (α and β). The microtiter plates are coated with anti-β hCG antibodies, which are exposed to the sample and europium-labeled anti-α hCG antibodies for 1 h. The wells are washed six times, and then a lanthanide chelator (enhancement solution) is added. The anti-estriol immunoassay is based on a competition between europium-labeled estriol and sample estriol for the binding sites of a limited number of anti-estriol antibodies conjugated to each well. After 1 h of incubation, the wells are washed six times, and then a lanthanide chelator (enhancement solution) is added. In all cases, the fluorescence emitted during each reaction was measured and recorded using the AutoDELFIA fluorometer.

The samples were contained in microtiter trays (96 wells) representative of each analysis, which included 12 wells devoted to calibration (duplicates of six calibrant concentrations); 3 wells spiked at anticipated low, medium and high levels; and 81 patient samples. The trays were collected shortly after AutoDELFIA analyses. The contents were degraded and stabilized on-site with the addition of 50 μL of 1:2 diluted HCl containing 5 ppb holmium (^{165}Ho). Before spiking, the wells were thought to contain ~200 μL of sample, and so the spiking yielded roughly 1:10 diluted HCl with ~1 ppb Ho. Normally, the Ho spike would be used by making up the sample to a known volume, after which the Ho allows internal standardization on the basis of its known concentration. Since the volumes of the samples were not explicitly known, the Ho spike must be considered as an internal standard related to the entire contents of each well.

The ICPMS instrument response to Eu and Ho was optimized and calibrated in 1:10 diluted HCl. The Eu response at each of its two isotopes (^{151}Eu, 47.8% abundance and ^{153}Eu, 52.2%) was ~15 cps/ppt, which was roughly one-half that of Ho (35 cps/ppt, monoisotopic ^{165}Ho). Plasma conditions were robust (high-temperature plasma) in order to minimize matrix suppression effects and to maximize the signals and are presented in Table 1.

The spiked microtiter trays were loaded into an autosampler. The sample was extracted at ~200 μL/min and nebulized using a PFA nebulizer into the ICPMS source. Five 3-s replicate measurements for each ^{151}Eu, ^{153}Eu, and ^{165}Ho were performed, and then the autosampler was directed to a wash solution containing 1:10 diluted HCl for 45 s. Data acquisition was synchronized with the well sampling.

Calibration curves were approximated using a linear fit (signal versus concentration for AFP and hCG; signal/maximum signal versus log concentration for estriol, where the "maximum signal" was taken as the average signal of the two zero-concentration standards) for both the AutoDELFIA and ICPMS analyses. The

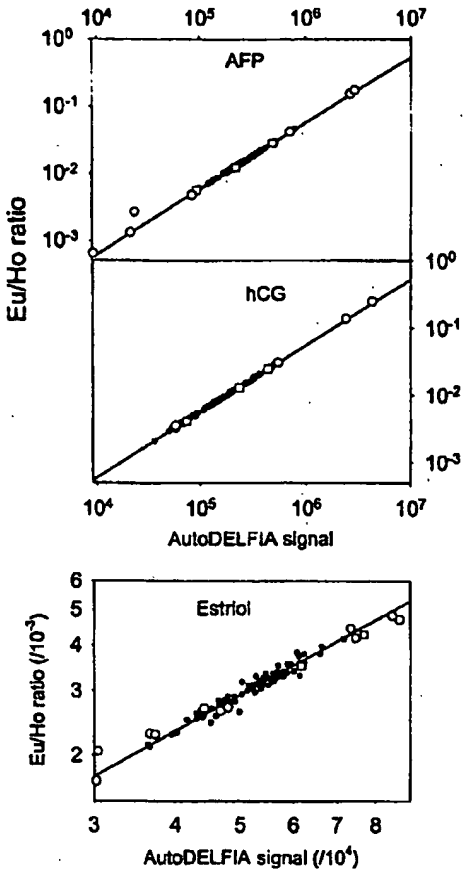

Figure 6. Eu/Ho signal ratio versus the AutoDELFIA signal for analysis of AFP, hCG, and estriol. The correlation coefficients (r^2) between Eu/Ho signal ratios and the AutoDELFIA signal for the detection of AFP, hCG, and estriol were 0.9997, 0.9998, and 0.976, respectively. Open circles, duplicate calibration standards; open squares, low, medium and high standards; closed circles, patient samples.

AutoDELFIA software constructs a calibration curve using spline fitting. The differences in quantitation results for the AutoDELFIA procedure and the linear fit were small in every case. To facilitate comparison, the linear fit calibration results were used in all cases in this work.

In all instances checked, the $^{151}Eu/^{153}Eu$ ratio was consistent with the expected natural abundance of europium, indicating that spectral interference was not significant. Further, the intensity of the Ho spike was similar to the calibration solution (1:10 diluted HCl) and showed a relative standard deviation of <7% in each microtiter plate run (which includes the effect of variations in sample volume). This suggests that matrix suppression due to the degraded antibodies, in the plasma and in the mass spectrometer was not observed for these samples.

The ICPMS results were obtained using both the absolute ^{153}Eu signal and the signal ratio $^{153}Eu/^{165}Ho$. The former yields a measurement of the concentration of the Eu tag in each well, and the latter is related to the absolute amount of Eu tag in each well. The normalization method also accounts for sample-to-sample variations in response, which may in part be due to differences in sample volumes.The Eu/Ho signal ratios correlate very well with the AutoDELFIA signals. The correlation coefficients (r^2), including all of the samples and standards, are presented in Figure 6. For each analyte, a graph presents the Eu/Ho signal ratio plotted against the AutoDELFIA signal. A linear fit sensitivity calibration for each analyte was used to derive a concentration for each of the patient samples. Because only two zero-concentration data points were provided for each analyte, the determination of the detection limit of the methods is difficult to establish. Using the 3-σ definition (see eq 1) and assuming that the standard deviation of duplicates is representative, the estimated detection limits of the methods are presented in Table 2. Although the detection limits for estriol appear comparable for the different methods, the AutoDELFIA method provides roughly 1 order of magnitude better detection limits for AFP and hCG than do the ICPMS methods, although it is based on a SD of only two blank measurements.

Table 2. Estimated Detection Limits of the Methods

analyte	estriol (nmol/L)	AFP (μg/L)	hCG (kU/L)
DELFIA	2.2.	0.018	0.00023
Eu/Ho ratio	2.5	0.22	0.0023

It can be inferred that the determination of the lanthanide tag by ICPMS provides precision and accuracy comparable to fluorescence determination according to the AutoDELFIA method. This is encouraging, because the tags have been optimized for fluorescence observation and not for mass spectrometry. It should be realized that the ICPMS method differs from the AutoDELFIA method only in the determination of the antibody complex following precipitation and separation from unreacted tag. Accordingly, the method inherits the benefits and problems of conventional immunoassay. Nevertheless, the AutoDELFIA antibody preparation process might be adapted to bind a larger number of lanthanide tag atoms, especially since the tag need not be fluorescent. In addition, the apparent ability to tag with different lanthanide atoms (elements or isotopes), perhaps in combination, offers intriguing opportunities for the simultaneous mass spectrometric determination of all three target analytes.

CONCLUSIONS

Immunoassay linked ICPMS determination of an elemental tag attached to the antibody offers several advantages over other methods:

- the tag is directly analyzed, implying the elimination of at least one step from the conventional immunoassay protocol;
- impurities have less impact, since they generally do not contain the target elements;
- nonspecific background is a constant fraction with time, unlike the instance of the ELISA assay in which the background depends on the incubation time;
- multiple tagging with a single isotope linearly improves the detection limit;
- it is reasonable to expect that multiple differently tagged antibodies can be used to probe one sample for simultaneous determination; and
- immediate acidification of the reacted and separated sample allows for long-term storage before analysis and simplifies assay protocols.

The ICPMS also brings particular attributes to the method that distinguish it from organic mass spectrometry (e.g., electrospray MS/MS):

- relative insensitivity to concomitant organic or metallic species;
- equivalent sensitivity to all similarly tagged antigen/antibody complexes;
- inherent ability to provide absolute quantitation;
- high sensitivity and low detection limits proportional to the number of similar atoms in the tag; and
- linearity of response.

The method remains limited by the constraints of specificity and reactivity associated with the immunoprecipitation process. Continuing efforts to alleviate the interference of nonspecific binding are required, and further improvements in sensitivity and efficiency of small volume flow rate sample introduction while retaining or improving washout would be welcome.

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